Video Article

Antibody Profiling by Luciferase Immunoprecipitation Systems (LIPS)

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Abstract

Technologies for comprehensively understanding and quantifying antibody profiles to autoantigens and infectious agents may yield new insights into disease mechanisms and may elucidate new markers to substratify disease with different clinical features and better understand pathogenesis. We have developed a highly quantitative method called Luciferase Immunoprecipitation Systems (LIPS) for profiling patient sera antibody responses to autoantigens and pathogen antigens associated with infection. Unlike ELISAs, the highly sensitive LIPS is easily implemented to survey humoral serological response profiles to different antigens in a universal format and produces dynamic antibody titer ranges up to 6 $\log_{10}$ for some antigens. In these studies, quantitative profiling by LIPS of patient humoral responses against panels of antigens or even the entire proteome of some pathogens (i.e. HIV), is typically more informative than testing a single antigen by ELISA. In addition, LIPS also eliminates time and effort needed to produce highly purified antigens as well as the labor-intensive assay optimization steps needed for standard ELISAs. Here we provide a detailed protocol describing the technical aspects of performing LIPS assays for readily profiling antibody responses to single or multiple antigens.

Protocol

1. Schematic Overview:

This video demonstrates the steps involved in performing the LIPS assay. Antigens are expressed in Cos1 cells as recombinant *Renilla* luciferase (Ruc)-antigen fusions, and crude extracts are obtained and used without purification. The LIPS assay is initiated by incubating crude Ruc-*antigen* extracts with patient sera in microtiter wells. The antibody-antigen mixture is then transferred to a 96-well filter plate containing protein A/G beads to capture IgG molecules. After washing the filter plate containing the protein A/G beads, antibody bound Ruc-antigen is measured by the addition of coelenterazine substrate and light units are measured with a luminometer.

PART 1: Transfection of Plasmids for production of *Renilla*-Antigen Fusion Proteins

Set-up: Cos1 cells are cultured in DMEM-10% FCS using standard tissue culture protocols. Plasmids for *Renilla* luciferase fusions have been described previously[1]. DNA for these plasmids is prepared using a Midiprep kit from Qiagen. The yield should be approximately 1 -3 mg. Measure the DNA concentration and store as a 1000 µg/ml stock solution at -20°C.

Procedure:

1. One day before transfection, split Cos-1 cells into new 100 x 20 mm dishes at approximately 2 X 10^6 per plate and incubate at 37 °C.
2. On the following day, the Cos-1 cells should be 80-95% confluent. Label 1.5 ml polypropylene microfuge tubes for each plasmid DNA to be transfected. Allow the FuGENE-6 transfection reagent, which is stored at 4° C, to warm up to room temp.
3. Add 94 µl of Opti-MEM media to each microfuge tube. Next add 6 µl of FuGENE 6 to the Opti-MEM media without touching the side wall.
4. Incubate the mixture for 5 minutes at room temperature.
5. Add 1-2 µg (from 1mg/ml DNA stock) of plasmid for *Renilla* luciferase antigen fusion construct. Mix and then incubate the mixture for 15 minutes at room temperature.
6. Transfer the DNA-FuGENE 6-Opti-MEM solution to the cells by dripping it evenly into the media of the Cos1 cells.

PART 2: Harvesting *Renilla*-antigen Fusions

1. Two days after transfection, the Cos-1 cells are harvested. This is initiated by removing the media and then rinsing the cells with 6 ml of PBS. After decanting the PBS, pipette away any residual PBS from the tissue culture dish.
2. Add 1.4 ml of cold lysis buffer composed of 50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 50% glycerol and protease inhibitors (2 tablets of complete miniprotease inhibitor cocktail per 50 ml of lysis buffer). Harvest cells with a cell scrapper and quickly transfer half of the lysate to each of two 1.5 ml microfuge tubes on ice.
3. A Branson Sonifier 150 is used to break the cells open. Place the microcentrifuge tube containing the cell lysate on ice and pulse for 5 sec, 5 sec and 5 sec with sonication settings of 2, 2 and 4, respectively.
4. Centrifuge the cell lysate at 12,500 RPM for two 4 minute spins at 4 ° C. After the first spin, gently invert the tubes to remove the loosely attached debris from the sidewall of the tube. After the second spin, carefully transfer the supernatant, without disrupting the pellet, from the two tubes to a new microfuge tube on ice.
5. Calculate the light units (LU) per µl of lysate. To measure the LU, dilute 1 µl of lysate with 8 µl of PBS in a new microfuge tube. Directly add 100 µl of 1X coelenterazine substrate to the diluted mixture and immediately measure luminescence in the tube using a tube luminometer (20/20^n Turner Scientific) with a 5 second read.
6. Store the Ruc-antigen lysate at -20° C for 1-2 days or store for longer period of times in aliquots at -80° C.

PART 3: Preparing a Sera Master Plate

1. Make a sera master plate by first adding 450 µl of buffer A (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100) to each well of a 96-deep-well polypropylene microtiter plate. At this step, a dye, Phenol Red, can also be included in buffer A (final concentration is 0.2 µg/ml in Buffer A) to act as a tracer for monitoring future sera sample addition and other steps of the LIPS assay.
2. Next add 50 µl of sera from each sample to the different wells containing 450 µl of buffer A. Note this is a 1:10 dilution of the sera in buffer A. Typically sera is not added to the last two wells of the master plate because this is reserved for the buffer blanks.
3. Before using the master plate, it is extensively shaken (1-2 hours) on a rotator platform. The serum in the master plate is stable for at least 1 month (or longer) at 4° C, if stored correctly to prevent evaporation.
4. As described below, this master plate provides 10 µl of diluted sera to be repeatedly removed for profiling of the sera against multiple antigens. Larger and smaller master plates can also be employed. Seal the plate well with Parafilm when it is not being used.

PART 4: LIPS assay

1. Polypropylene 96-shallow well microtiter plates are used to test sera. In the first step, add 40 µl of buffer A to each well of the 96-well plate using an 8 channel micropipette.
2. Next take 10 µl of diluted sera (1 µl sera equivalent) from the master plate and add it directly to each well of the working plate using an 8 channel micropipette.
3. A master mix containing the Ruc-antigen extract is next formulated such that 1 X 10^7 light units (LU) is added in 50 µl of buffer A to each well. Lower inputs (as little as 1 X 10^6) can also be used, but result in a lower dynamic range. Make this master mixture and pipette 50 µl of Ruc-antigen mixture to each well.
4. Incubate the plate on a rotary shaker for 1 hour at room temperature.
5. During the incubation, add 5 µl of a 30% suspension of Ultralink protein A/G beads (Pierce Biotechnology, Rockford, IL) in PBS to the bottom of each well of a new 96 well filter HTS plate (Millipore, Bedford, MA).
6. After the 1 hour incubation, transfer the 100 µl Ruc-antigen antibody reaction mixture to 96 well filter HTS plates containing the protein A/G beads using an 8 channel micropipette.
7. Incubate the 96-well filter plate on a rotary shaker for 1 additional hour at room temperature.
8. Next wash the filter plate on a vacuum manifold. Each well is washed 8 times with 100 µl of Buffer A, followed by two times with 100 µl of PBS. This can be performed manually or with a robotic pipetting workstation.
9. Following the last wash, the vacuum is turned off. Remove the filter plate and blot it dry using a stack of paper towels or filter paper making sure to remove moisture on the top and bottom of the plate.

PART 5: Measuring Luminescence and data analysis

Set-up:

A Berthold LB 960 Centro microplate luminometer is used for determining luminescence in each well using a single injector. Once the machine is on, rinse the injector with distilled H_2O using the injector wash cycle. Coelenterazine substrate is prepared using the Promega Renilla substrate kit as described by the manufacturer. Typically 6 ml of 1X coelenterazine substrate mix (i.e. 60 µl coelenterazine stock plus 6 ml of 1X buffer) is prepared for priming the machine and running one full 96-well plate. Before analyzing the plate, the Berthold LB 960 Centro microplate luminometer is primed with 1X coelenterazine substrate. Open a program file containing the setting for injecting the substrate and reading the plate. For these measurements, 50 µl of coelenterazine substrate is injected, the plate is shaken for 2 sec, followed by a 5 sec read of luminescence.

1. Although the plate luminometer has the capacity to read the entire plate, a partial read of the plate can also be selected (under the read menu). Start the program, which initiates reading of the plate.
2. After the run, remove the microtiter filter plate promptly to prevent spillage in the luminometer. Export the data generated with the MikroWin program into an Excel format for analysis.
3. We recommend evaluating the sera from two independent runs. Data is then averaged to generate the LU titer values for each sample. These values can further be adjusted to subtract the buffer blanks.
4. Additional data analysis such as determining the cutoff can be calculated by taking the mean plus 3 or 5 standard deviations of the control values.

Discussion

LIPS requires minimal assay optimization and, due to its simplicity, high quality data can typically be generated using LIPS in under two weeks for any given antigen. The most time consuming steps are cloning and generating the appropriate plasmid expression vector containing the Ruc-antigen fusion. Once these plasmids are generated, single or multiple antigens can be rapidly tested with LIPS as described above. It should be noted that LIPS is quite versatile such that sera can also be tested in a tube format or alternatively on a microtiter filter plate with the aid of a BIOMEK robotic workstation[2] or a plate washer with vacuum filtration. This highly scalable system allows for the parallel LIPS profiling of a panel of human autoantigens[3] or the whole proteome of a virus[4]. It is likely that many different antibody profiles generated by LIPS will be useful for diagnostics[3-9], antigen discovery[9], following the course of a treatment[10], vaccine monitoring and has broad implications for specific disease states and for public health in general.

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